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Feruloyl-L-arabinose attenuates migration, invasion and production of reactive oxygen species in H1299 lung cancer cells

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ABSTRACT

Ferulic acid (FA), a phenolic compound, is an abundant dietary antioxidant and exerts the mitogenic effect on cells. Recently, we isolated an active FA derivative, namely feruloyl-L-arabinose (FAA), from coba husk. The aim of this study was to investigate the effects of FAA on the proliferation, migration and invasion of H1299 human lung cancer cells. Our results showed a strong antioxidant potential of FAA. Additionally, FAA inhibited the migration and invasion ability, while causing a significant accumulation of G2/M-population, of H1299 tumor cells in a dose-dependent manner, whereas no significant change on cell proliferation was observed. Results from the wound healing assay revealed that cell migration ability was markedly inhibited by FAA treatments. Similarly, results of gelatin zymography study showed that FAA treatments significantly decreased the activities of matrix metalloproteinase (MMP)-2 and MMP-9, suggesting that FAA-mediated inhibition on migration and invasion of lung cancer cells may be achieved by the down-regulation of the MMPs activities. Taken together, our present work provides a new insight into the novel inhibitory function of FAA on cell migration in H1299 cells, suggesting its promising role in the chemoprevention of lung cancer.

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1. Introduction

Lung cancer is the leading cause of death in the world. The human non-small cell lung cancer (NSCLC) accounts for 80–85% of lung cancer cases. More than one of three patients with NSCLC is surgically unavailable (Pirker and Minar, 2010). Until now, chemotherapy is still the major approach in clinical NSCLC treatment (Ettinger et al., 2010; O'Rourke et al., 2010; Pirker and Minar, 2010; Wagner and Yang, 2010). Despite the continuing efforts on improving the current therapies and developing new regimens to treat lung cancers, both poor prognosis at the advanced stage of NSCLC and chemotherapeutic resistance contribute to the low survival rate of NSCLC patients (Wagner and Yang, 2010). Nowadays, numerous anticancer agents derived from natural products (Chiu et al., 2011; Liu, 2011; Wang and Yi, 2008) have been shown to exhibit improved chemopreventive effects against lung cancer, and this may shed the light on the efficacy of lung cancer therapy.

Ferulic acid (FA), or 4-hydroxy-3-methoxycinnamic acid (Fig. 1A), is an abundant aromatic natural constituent found in plant cell walls (Buanafina et al., 2010; Srinivasan et al., 2007). FA is known to exert potent effects on scavenging reactive oxygen species (ROS) and inhibiting lipid peroxidation, and thus has been approved as food additive to prevent lipid peroxidation in certain countries (Srinivasan et al., 2007). Recently, water-soluble FA sugar esters from wheat bran had been reported to stimulate the growth of *Bifidobacterium bifidum* (Yuan et al., 2005a), and effectively protect normal rat erythrocytes against AAPH-induced oxidative damage (Yuan et al., 2005b). In *in vitro* studies, water-soluble FA sugar esters were found to possess effective antioxidant activity towards low-density lipoproteins oxidation and exhibit better scavenging activity on 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals than FA (Ohta et al., 1997). In addition, water-soluble FA sugar esters exhibited protection capacity against oxidative damage due to

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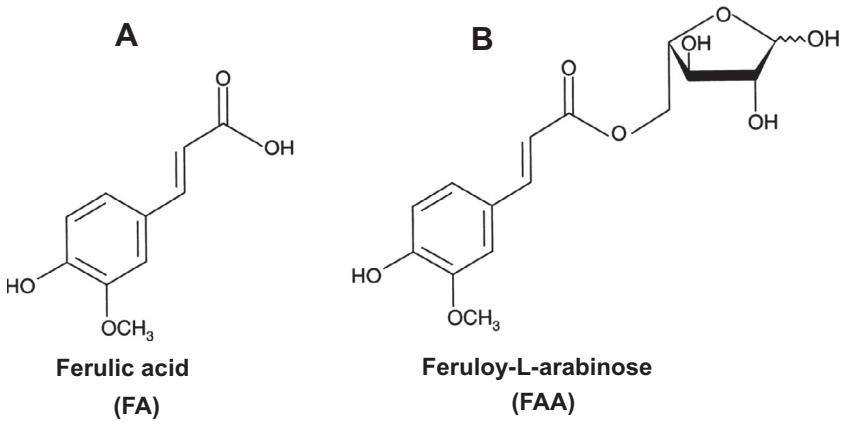

Fig. 1. Structures of ferulic acid (FA) and its derivative, Feruloyl-L-arabinose (FAA).

diabetes (Ou et al., 2007). Additionally, the major site of FA absorption is the colon, free FA and feruloyl-glucuronide could be efficiently transported through an *in vitro* model for the colonic epithelium consisting of co-cultured Caco-2 and mucus-producing HT29-MTX cells (Poquet et al., 2008). Based on these observations, FA and water soluble FA sugar esters demonstrated potential to be used as food additive for atherosclerosis prevention or other applications involving its antioxidant capacity.

Previous studies have shown that FA exhibited anti-proliferative effects on colon cancer cells (Janicke et al., 2011; Jayaprakasam et al., 2006), increased the radiosensitizing effects on cervical cancer cells (Karthikeyan et al., 2011) and showed the protective effects against chemical-induced DNA damages and/or carcinogenesis in various model systems (Stagos et al., 2005; Tanaka et al., 1993). In another study however, FA was shown to enhance proliferation of MCF7 and BT20 human breast cancer cells (Chang et al., 2006), indicating the possibility that FA might exhibit different biological effects depending upon cancer cell types it interacts with. However, the antioxidant activity of identified FA sugar esters in a cell model has not yet to be reported. Although FA and its various derivatives have been shown to exhibit the antioxidant effects *in vitro* and *in vivo*, they seem to possess diverse pharmacological functions, with some of which still remaining unknown. Therefore, the roles of FA derivatives in cancer cells remained to be fully investigated. To date, studies performed to investigate the direct effects of FA and its derivatives on lung cancer cells have been limited.

In the current study, we purified a FA derivative, feruloyl-L-arabinose (FAA) (Fig. 1B) from coba husk of plant *Zizania latifolia*, and examined its antioxidant ability on the lung cancer H1299 cell line as many cancers are known to possess elevated ROS levels that enhance their mobility, invasiveness and metastatic capabilities. H1299 cell is a large cell carcinoma, one subtype of non-small cell lung cancer cells, and has been reported to exhibit a high invasiveness compared to other lung cancer cells (Seo et al., 2009; Shatz et al., 2010). We hypothesized that FAA possesses anti-oxidant properties, causing the reduction of intracellular ROS that leads to decreased proliferation, altered cell cycle and reduced mobility in H1299 lung cancer cells. In addition, other effects of FAA, such as the scavenging ability of endogenous ROS, and influence on cellular invasion and proliferatory capacities, on H1299 cells were also examined.

2. Materials and methods

2.1. Preparation of FAA

Zizania latifolia coba husk was collected from local farm in Pu-Li, Taiwan. After washed and oven-dried at 60 °C for overnight, samples were milled and passed through a 0.5 mm sieve. Samples were defatted immediately using *n*-hexane with the Soxhlet apparatus. The dry defatted sample was then used for insoluble dietary fiber (IDF) preparation according to a previously published protocol (Bunzel et al., 2001). The mixtures of phenolic acid sugar esters were prepared from IDF of coba husk by chemical degradation with 0.2 M trifluoroacetic acid (TFA) at 100 °C for 115 min. The acid hydrolysate was then applied to an open column (20 cm × 7 cm diameter) packed with Amberlite XAD-2. Elution was successively performed with 2 volumes of distilled water, 2.5 volumes of 50% (v/v) methanol/water and 2 volumes of methanol. Considering of higher esterified ferulic acid content in the 50% (v/v) methanol/water fraction, this fraction was collected and concentrated for the isolation of phenolic acid sugar esters. Separation of phenolic acid sugar esters was achieved by means of further normal phase liquid chromatography with an open silica gel column (60 cm × 3 cm diameter) and semi-preparative RP-HPLC using a Inersile 10 ODS column (250 mm × 10.7 mm, VERCOPAK, Taiwan), and FAA was identified as the major product extensive spectrometric analysis (EI/MS and NMR) and by comparison with data reported in the literature (Saulnier et al., 1999). Proton and ^{13}C NMR spectra in D_2O were recorded at 599.95 and 150.87 MHz on a Varian Unity Inova-600 spectrometer locked on deuterated solvent and referenced to the solvent peak. Proton and ^{13}C NMR are relative to D_2O at $\delta = 4.8$ and the center line of CD_3OD at δ 49.0. The mass spectra (MS)-EI were recorded on a Finnigan TSQ Ultra EMR mass spectrometer with EI source. FAA was isolated as a light yellow powder with a molecular formula $C_{15}H_{18}O_8$ (EI-MS; $[M]^+$ 326) and NMR data (^1H NMR(599.94 MHz, D2O, 20 °C): δ FA, 3.7 (3H,s, –OCH3), 6.05 (1H, d, J = 16.2 Hz, –CHCHCOO), 6.7–7.9 (3H, m, aromatic), 7.3 (1H, d, J = 15.6 Hz, –CHCHCOO); L-Araf, 5.3 (1H, d, reducing proton),4.4–4.0 (4H, m, sugar protons). ^{13}C NMR (150.87 MHz, D2O, 20 °C): δ 169.06 [C-9]; 147.74 [C-4]; 147.41 [C-3]; 146.47 [C-7]; 126.71 [C-1]; 123.38 [C-6]; 115.32 [C-5]; 123.38 [C-6]; 113.50 [C-8]; 110.97 [C-2]; 101.37 [Araf-C1α]; 95.46 [Araf-C1β];81.37 [Araf-C2α]; 80.78 [Araf-C4α]; 78.75[Araf-C4β]; 76.20[Araf-C2β]; 76.05 [Araf-C3α]; 74.54[Araf-C3β]; 65.47 [Araf-C5β]; 64.17 [Araf-C5α]; 55.63 [C-10]) (Supplementary Fig. 1A and B). In all experiments in the study, FA and FAA was dissolved in DMSO (less than 0.1%) and H_2O, respectively.

2.2. Reagents

The following compounds were obtained from Gibco BRL (Gaithersburg, MD, USA): Dulbecco's modified Eagle's medium (DMEM), F-12 medium, fetal bovine serum (FBS), trypan blue, penicillin G and streptomycin. Phosphate-buffered saline (PBS), ribonuclease A (RNase A), propidium iodide (PI), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2′,7′-dichlorofluorescin diacetate (DCFDA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The WST-1 Cell Proliferation Assay kit was purchased from Roche Molecular Biochemicals (Rotkreuz, Switzerland).

2.3. Cell culture

Human non-small cell lung cancer (NSCLC) H1299 cell line was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Based on previously published protocols (Ballal et al., 2002; Wagner and Yang, 2010) with slight modification, H1299 cells were maintained in DMEM: F-12 medium (3:2 ratio) and supplemented with 8% FBS, 2 mM glutamine and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37 °C in a humidified atmosphere of 5% CO_2.

2.4. Determination of endogenous ROS

The changes in endogenous ROS levels were detected using the fluorescent indicator DCFDA (Sigma–Aldrich). A total of 1×10^5 H1299 cells were seeded onto a 6-well culture plate, treated with or without FAA for 24 h. Subsequently, attached cells were harvested and stained with 100 nM DCFDA in PBS for 30 min at 37 °C,

washed twice with PBS. The DCF fluorescence was measured by flow cytometry immediately. The measurement wavelengths for excitation and emission are 485 and 530 nm, respectively.

2.5. DPPH radical-scavenging activity assay

The anti-oxidant activities of FAA were measured based on the scavenging activity of DHHP free radical (Barreto et al., 2008; Blois, 1958). Briefly, vitamin C standards and various FAA concentrations were freshly prepared and diluted in methanol. Ten microliters of methanol (as blank control) or FAA solution was added to 90 μl DPPH solution (to yield final FAA concentration of 0.2 mg/ml) in a 96-well microplate. The mixture was mixed thoroughly and incubated at room temperature for 30 min in the dark. After incubation, the decrease in the solution absorbance was measured at 492 nm on a Multiskan Ascent 354 microplate reader (Thermo Fisher Scientific, Rockford, IL, USA). The DPPH radical scavenging activity was calculated as follows:

$$\text{DPPH radical scavenging activity } (\%) = (1 - A_0/A_1) \times 100$$

where A_0 and A_1 are the absorbance of the control and FAA, respectively. Each experiment was repeated three times and found to be reproducible within acceptable experimental errors.

2.6. Gelatin zymography

The activities of MMP-2 and MMP-9 were assessed by gelatin-zymography as previously described (Chiu et al., 2011) with minor modification. Briefly, 3×10^5 H1299 cells were seeded onto 12-well plates and cultured with various concentrations of FAA for 24 h. Cell lysate were prepared with standard SDS–PAGE loading buffer containing 0.01% SDS without β-mercaptoethanol and were not boiled before loading. The samples were subjected to electrophoresis with 10% SDS polyacrylamide gels containing 1% gelatin. The gels were thoroughly washed with distilled water containing 2.5% Triton X-100 to remove SDS. The gel was then incubated at 37 °C for overnight followed by staining with Coomassie brilliant blue R-250 and de-staining with methanol-acetic acid–water (50/75/875, v/v/v). The gelatinase activity was detected as the unstained gelatin-degradation zones within the gel. Signal intensities were analyzed using Gel Pro v.4.0 software (Media Cybernetics, Silver Spring, MD, USA).

2.7. Cell viability and proliferation assays

In brief, a total of 1×10^3 cells was seeded onto well in a 96-well plate and treated with PBS as vehicle or different FAA concentrations indicated for 24 h. After incubation, cell viability was determined by trypan blue dye exclusion assay combined with the Countess™ automated cell counter performed according to the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). Cell proliferation was assessed on cells treated with FAA for 24 h using a tetrazolium salt, 4-[3-(4-iodophenyl) 2-(4-nitrophenyl) 2H-5-tetrazolio] 1,3-benzene disulfonate (WST)-1, which produces a highly water-soluble formazan dye by the active dehydrogenase from live cells. The WST-1 assay was performed according to the manufacturer's instructions.

2.8. Cell cycle analysis

The cell cycle distribution was determined by propidium iodide (PI) staining as previously described (Chiu et al., 2010). In brief, 1×10^6 cells were treated with PBS (as vehicle control) or indicated FAA concentrations for 24 h. Cells were harvested with 0.25% trypsin, washed twice with PBS and fixed in 70% ethanol overnight. After centrifugation, the cell pellets were stained with 10 μg/ml PI (Sigma, St. Louis, MO, USA) and 10 μg/ml RNase A for 30 min at 37 °C in the dark. The samples were assayed using a FACScan flow cytometer (Becton–Dickinson, Mansfield, MA, USA) and the results were analyzed using the Cell-Quest software (Becton–Dickinson).

2.9. Wound-healing assay

The cellular migration was assessed as previously described (Chiu et al., 2011) with minor modification. A total of 3×10^5 H1299 cells were seeded onto 12-well plates and then grown to complete confluence. A 1000-μl plastic pipette tip was used to scratch the culture monolayer and create a clean 1-mm-wide wound area. Cells were treated with PBS (as vehicle control) or indicated concentrations of FAA. After further incubation at 37 °C for 16 h, the wound gaps were photographed and analyzed and calculated using the software "TScratch" (Geback et al., 2009).

2.10. Boyden's invasion assay

To further examine the effect of FAA on invasion ability of H1299 cells, the transwell invasion assay was performed as described previously (Tseng et al., 2009). Briefly, cells were seeded on the upper compartment of the Boyden's Chamber with serum-free medium. The 8-μm pore-size pores of the upper insert (ThinCert™, Greiner, Frickenhausen, Germany) are precoated with matrigel as basement matrix. The 10% FBS was added in the lower chamber as a chemoattractant. Cells were incubated with indicated concentrations of FAA for 12 h. The cells on the lower surface of the filter were paraformaldehyde-fixed, stained with Giemsa and counted.

2.11. Statistical analysis

All data are the means ± SD from at least independently triplicate experiments, with a three biological replicates per experiment. The significance of the differences was analyzed by a one-way analysis of variance (ANOVA) followed by a Dunnett's *t*-test correction, with $p < 0.05$ as considered significant.

3. Results

3.1. Radical-scavenging activity of FAA

To evaluate the radical scavenging activity of FAA, the DPPH assay was performed. The antioxidant activity of FAA was measured by assessing the capacity of FAA to scavenge DPPH. The absorbance in solution decreases upon accepting hydrogen atoms from an antioxidant. As shown in Fig. 2B and Supplementary Fig. 2, all concentrations (0.01, 0.03, 0.06, 0.15, 0.3 and 0.6 mM) of FA and FAA tested exhibited significant radical-scavenging activity as compared with the blank control. In addition, higher concentrations (0.15 mM and above) of FAA exhibited better radical-scavenging activity than FA (Fig. 2B)

3.2. FAA reduces the level of intracellular ROS

To investigate if FAA treatments affect ROS production, we examined intracellular oxidants by staining H1299 cells with DCFDA, which is oxidized by ROS to the highly fluorescent DCF. The DCF fluorescence was markedly diminished even at the lowest initial concentration (Fig. 3), suggesting that the effective ROS scavenge ability of FAA.

3.3. The effect of FAA on proliferation of NSCLC cells

To examine the effect of FAA on cell growth, H1299 cells were treated with PBS (as vehicle control) or different concentrations of FAA (ranging from final concentrations of 0.06–0.6 mM) for 24 h and 48 h, respectively, before gross morphological change was observed by light microscopy. Morphologically, there was no significant change observed between the vehicle control and FAA-treated cells (data not shown). Additionally, the cell survival was assessed by WST-1 proliferation assay and FAA was found to exert no significant cytotoxic effect on proliferation even at the highest dose (0.6 mM) (Fig. 4). Similarly, the trypan blue exclusion assay showed that the non-cytotoxic property of FAA (data not shown).

3.4. FAA causes a moderate accumulation of G2/M population

Next, we examined the effects of FAA on cell cycle progression in H1299 cells. With the increase of FAA concentrations, there was a significant drop in the G0/G1 phase, whereas a significant elevation of the G2/M population was also observed (Supplementary Fig. 3 and Table 1). The observation that FAA treatments caused a significant accumulation of the G2/M population but not inhibition on cell proliferation of H1299 cells suggested the non-cytotoxic property of FAA.

3.5. FAA attenuated migration of H1299 lung cancer cells

To investigate if FAA treatments would influence the migration of NSCLC cells, wound-healing assay and gelatin zymography were performed on H1299 lung cancer cells. It was shown that the

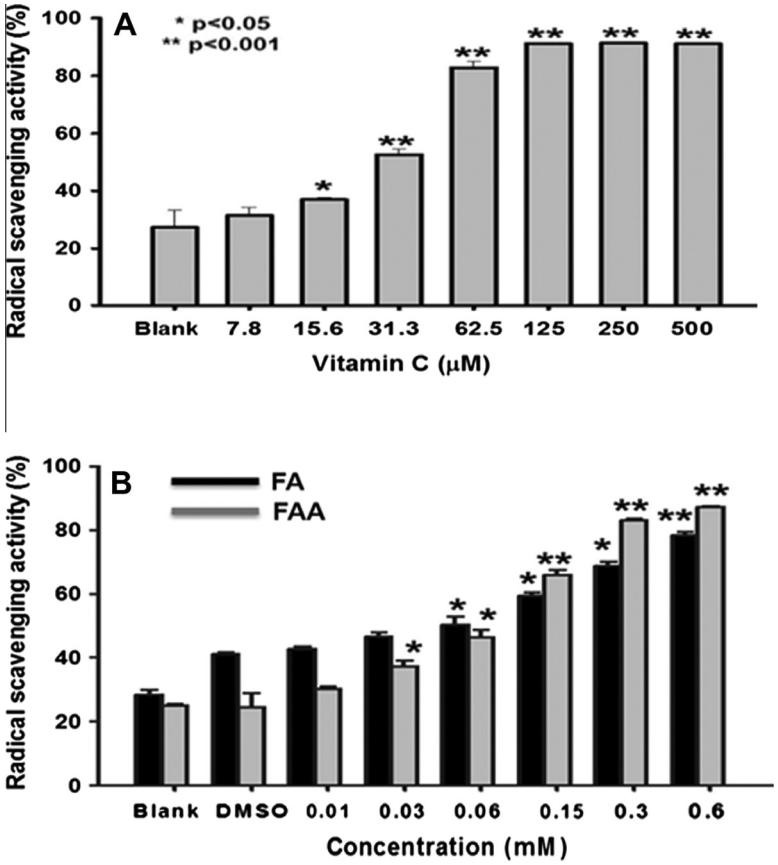

Fig. 2. DPPH radical-scavenging capacity of FA and FAA FAA was tested in an antioxidant assay by measuring the DPPH radical-scavenging activity. Vitamin C was serially diluted in methanol from 500 μM to 7.8 μM ($n = 3$) and as positive control. FA and FAA were diluted in methanol from 0.6 mM to 0.01 mM ($n = 3$) and incubated with DPPH as described in Section 2. The radical-scavenging capacities of FA and FAA at various concentrations were quantified as the percentage decrease of absorbance at 492 nm against the blank control. $^{*}p < 0.05$ and $^{**}p < 0.001$, respectively. Veh, vehicles for FA and FAA were DMSO and H_2O, respectively.

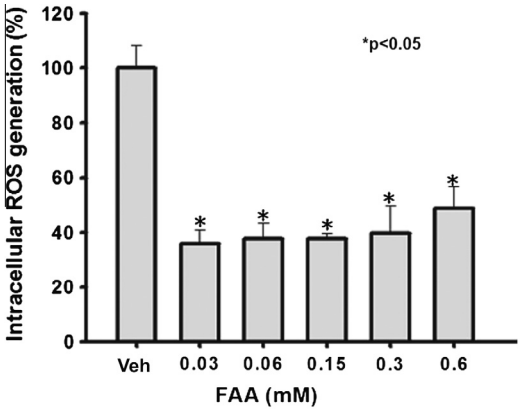

Fig. 3. FAA down-modulates the endogenous level of ROS in H1299 cells One hundred thousand H1299 cells were seeded onto a 6-well plate and treated with or without the indicated doses of FAA (0.03, 0.06, 0.15, 0.3 and 0.6 mM) 24 h. Afterwards, the reduction of endogenous ROS was determined by DCFDA staining combined with a flow cytometry analysis. $^{*}p < 0.05$ against vehicle.

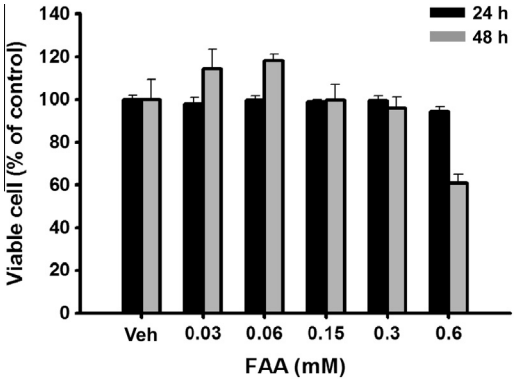

Fig. 4. Effect of FAA on proliferation of NSCLC cells One thousand H1299 cells were seeded onto a 12-well plate and treated with the indicated doses of FAA for 24 h and 48 h respectively. Cell proliferation was determined using the WST-1 assay described in materials and methods. Veh, vehicle control.

migration of H1299 lung cancer cells was inhibited by FAA in a dose-dependent manner, as demonstrated by the larger denude zones with increasing FAA concentrations at 16-h post-treatment (Supplementary Fig. 4A). Fig. 5A illustrates the quantitative analysis on the inhibition of migration ability by FAA treatments compared with the vehicle control. The calculated denuded zone (indicating the migratory ability of H1299 cells) for FAA concentrations used at 0.03, 0.06, 0.15, 0.3 and 0.6 mM was 90.86 ± 1.68%, 85.99 ± 1.35%, 83.96 ± 1.01%, 66.78 ± 1.77% and 56.88 ± 1.76% ($n = 3$), respectively. Likewise, the Boyden's chamber assay showed the anti-invasion potential of FAA (Fig. 5B and Supplementary Fig. 4B). In addition, as MMPs-mediated degradation of extracellular matrix plays an important role during tumor invasion, we used gelatin zymography to investigate the activities of MMP-2 and MMP-9 under a serum starvation condition of H1299 cells. After cells were treated with or without FAA for 24 h, a significant

Table 1
Effect of FAA on cell cycle progression of NSCLC cells H1299 cells were treated with the indicated doses, 0.03, 0.06, 0.15, 0.3 and 0.6 mM of FAA for 24 h. After flow cytometric analyses, the quantification analysis on distribution of cell cycle progression on FAA-treated NSCLS cells was performed. Data are presented as mean ± S.D. ($n = 3$). Different letter notations indicate the statistical significance between vehicle control and FAA treatment groups (a, no significance; a *vs.* b and a *vs.* c, statistically significant with $p < 0.005$ and 0.001, respectively).

%phase/FAA (mM)	Veh	0.03	0.06	0.15	0.3	0.6
Sub-G1	2.93 ± 0.3[a]	2.4 ± 0.2[a]	4.3 ± 0.2[b]	2.96 ± 0.1[a]	3.06 ± 0.4[a]	4.3 ± 0.5[b]
G0/G1	59.25 ± 0.4[a]	56.35 ± 0.7[b]	48.28 ± 0.1[c]	48.51 ± 0.6[c]	43.88 ± 0.6[c]	43.4 ± 0.1[c]
S	10.97 ± 0.5[a]	11.64 ± 0.4[a]	16.24 ± 0.8[c]	16.7 ± 0.3[c]	17.04 ± 0.3[c]	10.5 ± 0.3[a]
G2/M	26.85 ± 0.7[a]	29.61 ± 1.1[b]	31.18 ± 0.9[b]	31.83 ± 0.3[c]	36.02 ± 0.4[c]	41.8 ± 0.9[c]

[a] No statistical significance.
[b] $p < 0.05$.
[c] $p < 0.001$.

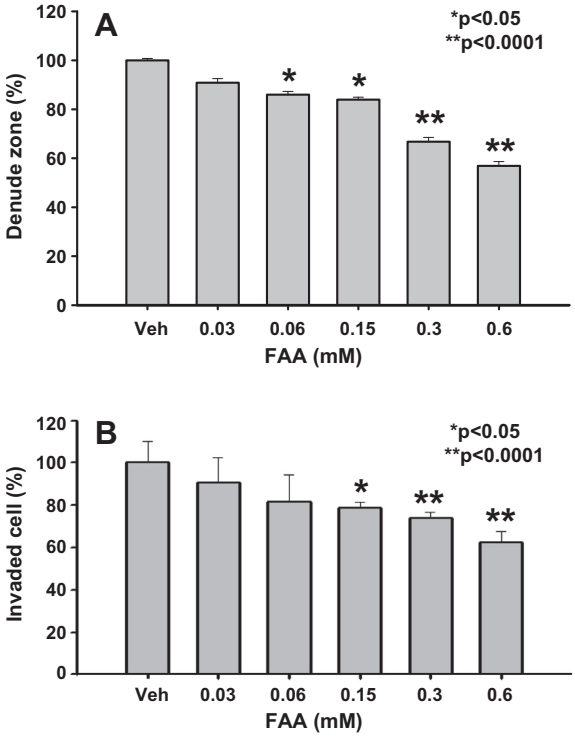

Fig. 5. Effect of FAA on migration of NSCLC cells FAA was test for its inhibition on migration of H1299 cells. (A) 5×10^5 cells were seeded onto a 12-well plate and scraped to create a clean 1-mm wide wound area within the confluent culture. Cells were treated with the indicated doses of 0.03, 0.06, 0.15, 0.3 and 0.6 mM of FAA for 16 h. Afterwards, the wound gaps were photographed using an inverted phase-contrast microscopy and quantified as compared to the DMSO vehicle control. $^{*}p < 0.0001$ vehicle versus FAA-treated. (B) The Boyden's transwell invasion assay showed that FAA significantly inhibited the cellular invasion of H1299 cells (12 h). $^{*}p < 0.05$ and $^{**}p < 0.001$ for FAA treatments against vehicle.

decrease in activation of both MMP-2 and MMP-9 was observed when compared with the untreated vehicle control (Fig. 6A and B). These results indicate that FAA inhibited the migration of H1299 lung cancer cells and it could be mediated at least in part by inhibiting activities of MMP-2 and MMP-9.c

4. Discussion

In our current study, we investigated the ability of FAA, an active FA derivative, to affect migration of lung cancer H1299 cells. Many chemopreventive and anti-cancer agents suffer from poor water-solubility, and therefore their further development in clinical therapeutics is limited. Similarly, the poor water solubility of FA (Maegawa et al., 2007), like other polyphenolic compounds, leads to its reduced bioavailability and may pose practical difficulties in its potential pharmacological applications, albeit the antioxidant potential it exhibits. A previous study (Mota et al., 2008) has demonstrated that the water solubility of FA was 0.57 ± 0.01 mg/ml and 2.19 ± 0.03 mg/ml at temperature 23 °C and 49 °C, respectively, in the acidic environment (pH < 3.5). Compared to the hydrophobicity of FA, we observed the feasible solubility of FAA (>10 mg/ml in water or PBS, unpublished data) at neutral pH value, indicating that FAA exerts a good hydrophilic property. The addition of mono-saccharides (such as arabinose, galactose and xylose) has been shown to improve the water-solubility of hydrophobic compounds (Gray et al., 2003; Wen et al., 2011). Accordingly, our data suggested that the presence of arabinose may contribute to sound water solubility of FAA, which would be very useful when FAA is applied to future biological analysis and applications.

As expected, the DPPH free-radical scavenging assay demonstrated that the strong antioxidant ability of FAA was comparable to FA (Fig. 2B). However, many compounds which exert *in vitro* anti-oxidative properties often up-regulate the ROS levels in cells in a dose-dependent manner (Jagdeo et al., 2010). In lung cancers, the large cell carcinoma exerted a high invasiveness compared to other NSCLC tumor cells (Seo et al., 2009; Shatz et al., 2010). Intracellular ROS, such as superoxide, has been shown to serve as a second messenger for signal transduction and signal amplification (Reth, 2002). ROS were reported to be essential for proliferation, adhesion and migration of cells (Huo et al., 2009; Shimizu et al., 2009; Vermeij and Backendorf, 2010). For example, Huo's work showed that ROS are critical mediators in epidermal growth factor (EGF)-induced proliferation and migration of corneal epithelial cell (Huo et al., 2009). As many cancers are known to possess elevated ROS levels that enhance their mobility, invasiveness and metastatic capabilities, we were intrigued to evaluate whether FAA would modulate the intracellular ROS levels in H1299 lung cancer cells. Our results showed that generation of intracellular ROS was significantly reduced in H1299 cells in a dose-dependent manner with all FAA concentrations tested (Fig. 3).

To further evaluate the effects of FAA, other cellular parameters, including changes in cell morphology, viability, migration and cell cycle distribution were also performed in NSCLC H1299 cells. Our results showed that no significant changes in either cell proliferation or morphology were observed in FAA-treated H1299 cells (Fig. 4 and data not shown). Additionally, in the higher FAA concentration (0.6 mM) used, it exerted no significant cytotoxicity towards H1299 cancer cells as well as the normal lung fibroblast MRC-5 cells (data not shown). This indicated that FAA might possess the potential of serving as an anti-oxidative agent in clinical or chemopreventive applications without inducing significant side effects.

While FAA treatments did not elicit noticeable cytotoxicity, we found that a significant G2/M accumulation was observed in FAA-treated H1299 cells in a dose-dependent manner in a cell cycle distribution analysis (Table 1 and Supplementary Fig. 3). Although p53 has been considered to be the major factor for driving G2/M

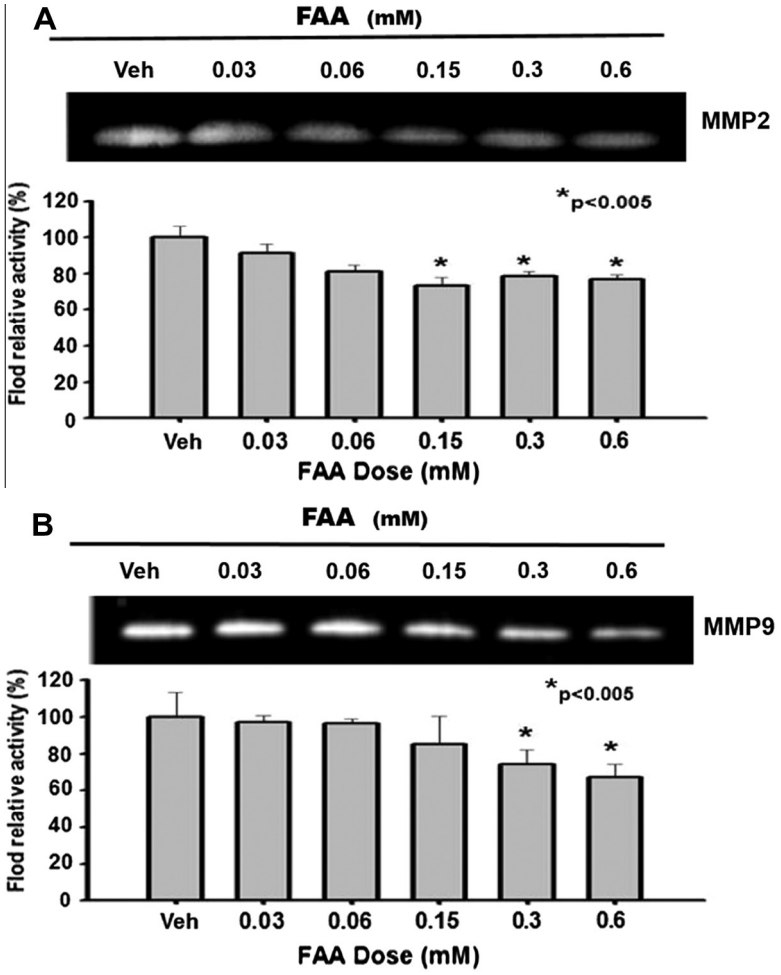

Fig. 6. Effect of FAA on activities of MMP-2 and MMP-9 After H1299 cells were treated with or without different concentrations of FAA for 24 h, the activities of (A) MMP-2 and (B) MMP-9 were detected by gelatin zymography ($n = 3$). Representative results are shown here. $^{*}p < 0.005$ for FAA treatments against vehicle.

cell cycle arrest, some alternative signaling events may drive G2/M accumulation in p53-deficient cancer cells. For example, 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced p21 expression causes the G2/M arrest is p53-independent in human breast carcinoma MDA-MB-231 cells. Additionally, a chaperone inhibitor, 17-allylamino-17-demethoxygeldanamycin (17AAG), causes G2/M arrest in p53-defective cells by down-regulating two checkpoint kinases, Chk1 and Wee1 (Tse et al., 2009). Likewise, our previous study showed that Etoposide, a topoisomerase inhibitor, induced G2/M accumulation via p21 in p53-deficient H1299 cells (Chiu et al., 2005b). Therefore, it is speculated that FAA may similarly modulate the accumulation of G2/M phase via p21 or other p53-independent pathway in H1299 cells. However, the detail mechanism needs to be further examined.

During cancer angiogenesis, MMPs play a pivotal role by helping degrade structural proteins surrounding blood vessels, softening the vessel walls and accelerating the formation of new capillaries via the escape of endothelial cells into the interstitial matrix. Elevated levels of both MMP-2 and MMP-9 have been demonstrated with poor prognosis and metastasis in patients with lung cancer (Leinonen et al., 2008; Qian et al., 2010; Peng et al., 2012). Additionally, it has been documented that a higher accumulated ROS is often detected in cancer cells, and the intrinsic ROS induces activation and release of MMPs, especially MMP-2 and -9 (Wu, 2006). Since MMPs-mediated degradation of extracellular matrix plays an important role during tumor invasion, we investigated if FAA treatments would cause change of activities of MMP-2 and MMP-9 in H1299 lung cancer cells. Our zymographic results demonstrated that FAA treatments, when used at 0.15 mM (for MMP-2) and 0.3 mM (for MMP-9) or above, can significantly reduce activities of respective MMPs in H1299 lung cancer cells (Fig. 6). This suggests that this inhibitory ability of FAA on MMP-2 and MMP-9 might contribute to reduced migration of H1299 human lung cells. Therefore, it is strategically possible to inhibit cancer metastasis via lowering the intracellular ROS levels in cancer cells. Recently, it has been demonstrated that FA inhibits UV-induced expression of MMP-2 and MMP-9, the major causes for skin aging in mouse skin (Staniforth et al., 2012), suggesting the protective effect of FA on photo-aging and photo-pathogenesis of skin. Their finding is consistent with our zymographic analyses on activities of MMP-2 and MMP-9. Consistent with these previous studies, the reduction of intracellular ROS might be the cause of attenuated MMP-2 and -9 activities, leading to the inhibition on migration of H1299 lung cancer cells (Fig. 5).

It has been shown that drugs capable of enhancing the generation of intracellular ROS can cause G2/M arrest in various cancer cells (Hong et al., 2012; Hseu et al., 2012). However, only few studies reported the effects of ROS scavenger on cell cycle distribution. Recently, it was reported that grape antioxidants caused a G1-phase arrest and may be involved in up-regulation of $p21^{cip1}$ and $p27^{kip1}$expression in oral cancer cells (Lin et al., 2012). It has also been shown that the antioxidant properties of *Liriodendron tulipifera* extract induced G2/M arrest in colon cancer cells (Wang et al., 2012). Accordingly, Lin's observation is consistent with the cell

cycle analysis in our current study, indicating the possible effect of ROS scavenger, such as FAA, on modulating cell cycle distribution.

Previous studies showed that FA protects cells from nicotine-induced DNA damage and cellular changes in cultured rat lymphocyte and rat (Sudheer et al., 2008, 2007), indicating the protective role of FA on cells. More recently, Janicke's work showed that FA may contribute to the protective effect cells via modulating the progression of S-phase in colon cancer cells Caco-2 (Janicke et al., 2011). Additionally, data from various studies have demonstrated the anticancer effects of FA and its derivatives. For example, Cione's work indicated that synthesized FA induced the release of cytochrome c from rat testes mitochondria (Cione et al., 2008). Additionally, Balakrishnan's study demonstrated the protective role of FA on a carcinogen 7,12-dimethylbenz[a]anthracene (DMBA)-induced abnormal expression of p53 and bcl-2 proteins in the buccal mucosa of hamsters (Balakrishnan et al., 2010). In comparison with FA and other FA derivatives, our study showed that FAA exerted non-cytotoxic effects towards H1299 lung cells as well as normal fibroblasts (unpublished data). In addition, the wound healing assay showed that FAA significantly attenuated migration ability of NSCLC H1299 tumor cells, indicating its potential effect on the metastasis of lung cancer cells.

Very little was known regarding the effect of FA on lung cancers in past studies. Recently, Bandugula's work demonstrated that 2-Deoxy-D-glucose (2-DG), a glycolytic inhibitor combining FA sensitized NSCLC H460 cells towards irradiation treatment (Bandugula and Rajendra Prasad, 2013). Furthermore, the enhanced anti-proliferation and apoptosis-inducing effect by co-treatment of 2DG and FA may act through up-regulating the expression of pro-apoptotic p53 in H460 cells (Bandugula and Rajendra Prasad, 2013). More than 50% of NSCLC cells were reported to be p53 mutated (Winter et al., 1992), and the p53 mutation was thought to contribute to chemoresistance and poor prognosis of cancer patients (Lin and Beer, 2012; Viktorsson et al., 2005). Interestingly, our results showed that FAA treatment inhibits the cellular migration and invasion of H1299 which was p53-deficient, suggesting that FAA could exert anti-cancer effect via p53-independent mechanism, and FAA application may provide a promising advantage on treating p53-deficient lung cancers.

In summary, results from our current study suggested that FAA is a potential antioxidant and is able to inhibit MMP-mediated lung cancer progression. To our best knowledge, this is the first research demonstration that FAA dramatically attenuated the migration ability of cancer cells by inhibiting MMP-2 and MMP-9 activity. In agricultural economics, FAA used in the study was isolated from coba husk which is one of the most abundant agricultural wastes in Taiwan. Therefore, the FAA production will greatly improve the economic value of coba husk. Accordingly, FAA could be a potential candidate of chemopreventive agent with good water solubility. Therefore, further investigation on the anti-tumor effects of FAA on other lung cancer cell models is warranted for the future development of treatment and chemoprevention for lung cancer.

Conflict of Interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fct.2013.05.019.

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